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Expression of all six human Torque teno virus (TTV) proteins in bacteria and in insect cells, and analysis of their IgG responses ☆

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ABSTRACT

Torque teno virus (TTV) is a non-enveloped human virus with a circular (~3800 nt) ssDNA genome. TTV transcription results in three viral mRNAs and six proteins, the function or antigenicity of which are unknown.

The six open reading frames of TTV genotype 6 were expressed in bacteria and insect cells. Expression of the ORF1/1-encoded protein was inefficient, while expression of the others was successful, with ORF1 and ORF1/2 as arginine-rich region depleted.

All six recombinant TTV proteins were antigenic. Of healthy adults, 11/25 (44%) showed strong IgG reactivity with one or more proteins. Four subjects, two of whom were genotype-6-DNA positive, were followed. One of the latter showed concurrently a strong IgG response against the ORF1 protein. The other showed appearance of IgG against the ORF2 protein concomitantly with resolution of the genotype-6 viremia. The genotype-6 sequences remained unaltered for years, suggesting that some mechanisms other than amino acid substitutions play a role in TTV immune evasion.

Introduction

Torque teno virus (TTV) was found in 1997 from a Japanese patient with hepatitis of unknown etiology (Nishizawa et al., 1997). TTV is a small, non-enveloped virus containing an approximately 3.8 kb long, circular negative-sense single-stranded DNA genome (Miyata et al., 1999; Mushahwar et al., 1999). By genomic organization, TTV resembles the chicken anemia virus (CAV) of the *Circoviridae* family, and is currently classified as a member of a new, floating genus *Anellovirus* (Biagini et al., 2004).

TTV shows a very high sequence variation, both at nucleotide and amino acid levels. Several genotypes (differing by more than 30%) have been identified, and form five major phylogenetic clusters (Biagini et al., 2004). Also other TTV-like viruses have been characterized: Torque teno minivirus (TTMV) (Takahashi et al., 2000) and Torque teno midivirus (TTMDV) or small anellovirus (SAV) (Jones et al., 2005; Ninomiya et al., 2007a). All these viruses have genomes of similar structure, yet with different sizes: 3.7–3.8 kb for TTV, 3.2 kb for TTMDV/SAV, and 2.8–2.9 kb for TTMV (Biagini et al., 2007; Jones et al., 2005; Ninomiya et al., 2007a; Takahashi et al., 2000).

Nearly all TTV research has been hitherto based on the detection of viral DNA by PCR. It has become evident that this virus is spread worldwide, with a ~90% DNA prevalence in blood of asymptomatic individuals (Abe et al., 1999; Huang et al., 2001; Kakkola et al., 2002; Okamoto et al., 1999; Simmonds et al., 1999). Persistent infections and co-infections with several genotypes are common (Ball et al., 1999; Biagini et al., 1999; Irving et al., 1999; Lefrere et al., 2000; Sugiyama et al., 1999). In addition, co-infections have been shown to occur with all three TTV-like viruses in asymptomatic subjects (Biagini et al., 2006; Ninomiya et al., 2007b).

The TTV genome consists of a 2.6 kb coding and 1.2 kb non-coding region, the latter containing a GC-rich region, a promoter and transcriptional enhancer elements (Kamada et al., 2004; Miyata et al., 1999; Mushahwar et al., 1999; Suzuki et al., 2004). A TATA-box and a poly-A sequence define the coding area, in which overlapping open reading frames (ORF) in all three frames are located (Erker et al., 1999; Hijikata et al., 1999; Miyata et al., 1999). TTV has been shown to produce by alternative splicing three mRNA species (Kamahora et al., 2000), from which by alternative translation initiation six proteins are produced (Qiu et al., 2005). The splice sites are well conserved among various isolates (Peng et al., 2002). Even though variation between the TTV genotypes is higher at the amino acid than at the nucleotide level (Tanaka et al., 2000), the proteins encoded by different genotypes have similar motifs, thus suggesting similar functions.

☆ Nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession number AY666122.

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The longest open reading frame, ORF1, has been suggested to encode a capsid protein (Takahashi et al., 1998). ORF1 has circovirus-like replication-associated motifs (Erker et al., 1999; Tanaka et al., 2001) and a CAV-like arginine-rich N-terminus (Mushahwar et al., 1999; Okamoto et al., 1998; Takahashi et al., 1998) that has been suggested to have DNA-binding activity and to function in packaging of the viral DNA (Erker et al., 1999). ORF1 contains hypervariable regions in which mutations leading to amino acid changes occur more frequently than in the remaining part of the protein (Jelcic et al., 2004; Nishizawa et al., 1999), suggesting immune evasion by hypermutations.

ORF2 encodes a putative 200-amino-acid protein that has a conserved amino acid motif (Hijikata et al., 1999; Peng et al., 2002). This motif is part of the dual specificity phosphatase sequence, the activity of which has been shown also in CAV and TTMV proteins (Peters et al., 2002). In several genotypes a stop codon divides ORF2 into two smaller frames, ORF2a and ORF2b. Even though the ORF2b protein is less conserved than the ORF2a at the amino acid level, it contains a CAV-like motif (Kakkola et al., 2002; Tanaka et al., 2000; Ukita et al., 2000). Very recently the ORF2 protein was shown to suppress the NF-κB pathway and thus to have putative regulatory effects on innate and adaptive immunity (Zheng et al., 2007).

The genotype-1a ORF3 protein [corresponding to ORF2/2 in (Qiu et al., 2005), and ORF2-4 in (Kamahora et al., 2000)] has similarities with a non-structural protein NS5A of hepatitis C virus (HCV). In Cos-1 cells, this protein is produced in two forms, of which the slower-migrating is phosphorylated at the C-terminal serine residues (Asabe et al., 2001). The remaining three TTV proteins are essentially unknown, as are the functions of all the six TTV proteins.

Only a few publications exist on TTV immunology and on the expression of TTV proteins. Tanaka et al. (2000) expressed by an *in vitro* transcription/translation method the putative ORF2 proteins, showing the functional stop codon dividing the ORF2 into smaller frames (Tanaka et al., 2000). ORF3 of genotype 1a was expressed for phosphorylation studies in Cos-1 cells (Asabe et al., 2001). For immunological studies, the putative capsid protein encoded by ORF1 has been produced in fragments. Lo et al. (1999) expressed in prokaryotes ~120 amino acids of ORF1 (the N22 region within the C-terminal half), and found by immunoblots no IgG antibodies in asymptomatic adults (Lo et al., 1999). Ott et al. (2000) expressed in prokaryotes the C-terminal (aa 504–752) and Handa et al. (2000) the N-terminal (aa 1–411) part of genotype-1 ORF1. These two groups found by immunoblots antibodies in 98.6% of hepatitis patients, blood donors, and asymptomatic children, and in 38% of blood donors, respectively (Handa et al., 2000; Ott et al., 2000). We have previously expressed in prokaryotes the two ORF2 proteins, ORF2a and ORF2b, and detected IgM and IgG in 9–10% of asymptomatic adults (Kakkola et al., 2002). In addition to immunoblotting, the existence of TTV antibodies in human sera has also been shown by other methods, including immunoprecipitation and immunocapture combined with PCR (Tsuda et al., 1999; Tsuda et al., 2001).

As the methods in current use for detection of TTV infections (restricted to acute and persisting, but not past) are PCR-based, little is known of the TTV proteins and their antigenic potential. Serological tests (measuring also past infections) would give valuable information on immunological reactions in humans against this highly prevalent, persisting, and potentially non-pathogenic virus.

In our previous studies we have cloned in full length the genome of TTV genotype 6 (Kakkola et al., 2007), and have identified the proteins encoded (Qiu et al., 2005). The aim of the current study was to express all the six TTV proteins in prokaryotic or baculoviral expression systems for use as antigens in immunological and diagnostic studies.

Results

Expression of the proteins in bacteria

Of the six protein coding regions (Fig. 1), altogether 11 constructs were cloned into bacterial expression plasmids: ORF1ΔArg, ORF1-N, ORF1-NΔArg, ORF1-C, ORF2, ORF2/2, ORF2/3, ORF1/1, ORF1/ΔArg, ORF1/2 and ORF1/2ΔArg. The proteins were expressed in fusion with GST in BL21 cells, and were analyzed by immunoblotting with GST antibody. The Rosetta strain was attempted with the arginine-rich proteins ORF1-N, ORF1/1 and ORF1/2, and with one arginine rich region-depleted protein ORF1/1ΔArg, however, with no improvement in expression levels. The successful expression conditions for each construct are given in Table 1.

Since the expression of the arginine-rich ORF1 protein was unsuccessful in our previous study (Kakkola et al., 2002), we decided to express the protein in bacteria as arginine-depleted constructs (ORF1ΔArg, ORF1-NΔArg) or in two parts (ORF1-N, ORF1-C). The expressions of the ORF1-NΔArg (~63 kDa) and ORF1-C (~69 kDa) proteins were successful, as was the expression of ORF1ΔArg (~100 kDa), however, shorter forms of the latter protein were also produced (Fig. 2). The expression of ORF1-N (~70 kDa) was not successful with any of the three media regardless of induction time. For this construct only the Rosetta strain was used.

The expression of ORF1/1 (~54 kDa) was of low level with any of the three media, with any of the induction times, and with both of the bacterial strains. Removal of the arginine-rich part (ORF1/1ΔArg; ~47 kDa) did not significantly improve the expression (Fig. 2). The expression of ORF1/2 (~44 kDa) was likewise low in any of the expression conditions; however, the removal of the arginine-rich part (ORF1/2ΔArg; ~37 kDa) resulted in major improvement of expression level (Fig. 2).

In our previous study we had expressed the fp2a (nt 104–253) and fp2b (nt 237–707) proteins from the ORF2 coding area (Kakkola et al., 2002). The ORF2 (nt 354–707; ~44 kDa) encoded protein expressed in this study corresponds to amino acids 40–118 of the previously expressed fp2b, and was successfully expressed in prokaryotes (Fig. 2). Also the expressions of ORF2/2 (~57 kDa) and of ORF2/3 (~56 kDa) were successful (Fig. 2).

For expression of some constructs, room temperature and IPTG concentration of 0.2 mM were also tried, but with no significant improvement. However, with the constructs that were prone to forming shorter fragments in standard conditions, shifting of the growth/induction time e.g. from 4/2 to 2/4 h brought a substantial gain in protein yield (less fragmentation; data not shown).

Expression of the proteins in insect cells

Of the six protein coding regions, altogether 7 constructs, covering all the reading frames in the coding area, were cloned into a

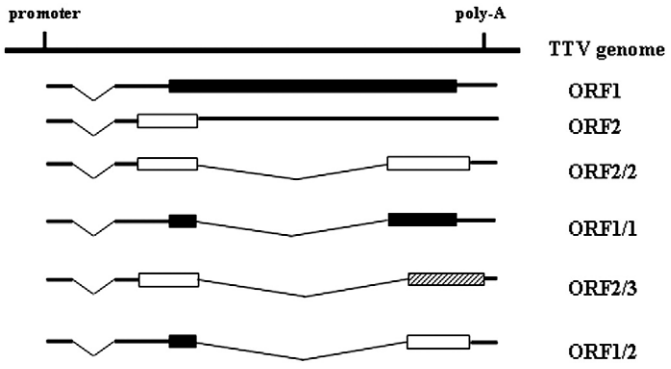

Fig. 1. Schematic presentation of the transcription map of TTV genotype 6 (adapted from Qiu et al., 2005). Thin lines represent introns, thick lines mRNA, and boxes translated areas, the shading of which indicate different reading frames.

Table 1
Expression conditions used in prokaryotes for the TTV protein constructs

	ORF1ΔArg	ORF1-NΔArg	ORF1-C	ORF2	ORF2/2	ORF2/3	ORF1/1	ORF1/1ΔArg	ORF1/2	ORF1/2ΔArg
Dilution[a]	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:100	1:50
Growth medium[b]	TF	LB	YT	LB	LB	LB	TF	YT	TF	LB
Growth/induction time in hours[c]	5/2	6/1	5/2	4/2	4/2	4/2	5/2	5/2	5/2	2/4

[a] Dilution of over night grown bacteria for induction of expression.
[b] TF = Terrific Broth, LB = L-Broth, YT = Yeast Extract Tryptone medium.
[c] e.g. 5/2 = bacteria grown for 5 h after dilution and subsequently induced (for expression) for 2 h.

baculoviral expression plasmid: ORF1, ORF1ΔArg, ORF1-N, ORF1-C, ORF2/2, ORF2/3 and ORF1/1. The proteins were expressed as GST-6xHis-fusion proteins, and were analyzed by immunoblotting, first with the GST antibody.

The expression levels of ORF1-N (~70 kDa) and ORF1/1 (~54 kDa) were of low level, while the expression of full-length ORF1 (~110 kDa) was unsuccessful. The expression of ORF1ΔArg (~100 kDa), ORF1-C (~69 kDa), ORF2/2 (~57 kDa) and ORF2/3 (~56 kDa) were successful and yielded adequate amounts of fusion proteins (Fig. 3).

IgG responses in human sera

To study the IgG seroprevalences in 21 humans, we used the best protein constructs, i.e. the bacterially expressed ORF1-NΔArg, ORF1-C, ORF2, ORF2/2, ORF2/3 and ORF1/2ΔArg, and the insect cell-derived ORF1ΔArg, ORF1-N, ORF1-C, ORF2/2, ORF2/3 and ORF1/1. Follow-up sera additionally obtained from 4 individuals were studied with the same constructs, and with the bacterially expressed ORF1ΔArg and ORF1/1ΔArg.

A sample was regarded as IgG positive only with an unequivocal band of correct molecular size in immunoblot. Entirely negative immunoblots were regarded as negative. Faint or barely visible bands, or inconsistent results, were considered borderline (+/−).

The seroprevalences and the corresponding TTV DNA prevalences (obtained with UTR-PCR) are shown in Table 2. Altogether 11/25 (44%) subjects showed positive and 16/25 (64%) showed borderline reactivities with one or more of the expressed proteins. A representative sample is shown in Fig. 4. Of the 21 individuals (excluding follow-up subjects), 5 (24%) were negative for all TTV antigens. The prokaryotic and eukaryotic antigens gave similar results.

Of the 21 individuals, 18 were studied for each of the four ORF1 constructs. The remaining three individuals were studied only for the bacterially expressed ORF1-C, and one of them also for ORF1ΔArg. If any of the expressed ORF1 constructs showed positive or borderline results, the sample was classified as ORF1-IgG positive or as borderline, respectively. Of the 21 subjects, 3/21 (14.3%) were ORF1-IgG positive, whereas 11/21 (52.4%) were negative (Table 2). Interestingly, all the ORF1 responses were towards the ORF1ΔArg and ORF1-C proteins, and none towards ORF1-N (with or without the arginine-rich region).

For the other proteins, positive responses were detected towards the products of ORF2 in 6/21 (28.6%), ORF2/2 in 1/19 (5.3%), ORF2/3 in 5/21 (23.8%), ORF1/1 in 1/18 (5.6%), and ORF1/2 in 0/20 (0%) of the subjects (Table 2). The 12 subjects (excluding those followed up), who showed positive ($n=6$) or borderline ($n=6$) IgG responses towards the ORF2-encoded protein, were compared with those of IgG responses towards fp2a and fp2b in our previous study (Kakkola et al., 2002). All eight fp2b seropositive subjects identified previously were in the current study classified as ORF2 IgG positive or borderline, and four additional subjects showed borderline IgG responses. Of the three individuals who had shown IgG towards fp2a, one was ORF2 IgG negative, and two, who also had IgG towards fp2b, were ORF2 IgG positive.

Of the 21 subjects, 17 were tested by PCR for genotype-6 DNA in serum, and two (11.8%) were found positive. Of these two, subject #23 showed IgG reactivity with ORF2, ORF2/2 and ORF2/3, was borderline with ORF1-C, and was seronegative for ORF1/2 (the other constructs

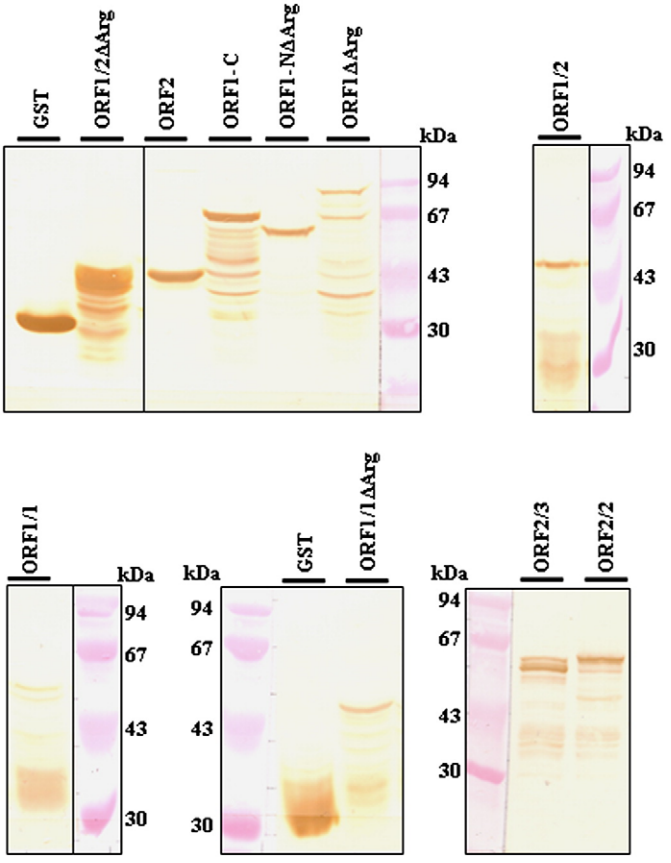

Fig. 2. TTV proteins expressed in bacteria. Immunoblotting with anti-GST antibody.

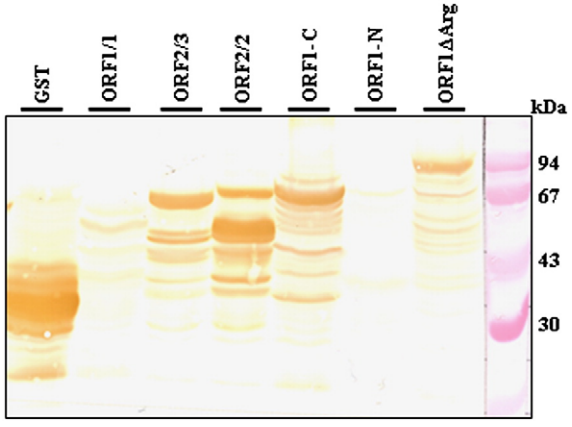

Fig. 3. TTV proteins expressed in insect cells. Immunoblotting with anti-GST antibody.

Table 2
IgG seroprevalences with each TTV recombinant protein in TTV-DNA-positive and negative subjects (by UTR PCR)

	IgG positive	IgG borderline	IgG negative	Total
IgG for ORF1[a]				
PCR+	2/21 (9.5%)	6/21 (28.6%)	10/21 (47.6%)	18/21 (85.7%)
PCR−	1/21 (4.8%)	1/21 (4.8%)	1/21 (4.8%)	3/21 (14.3%)
Total	3/21 (14.3%)	7/21 (33.3%)	11/21 (52.4%)	21/21 (100%)
IgG for ORF2				
PCR+	6/21 (28.6%)	5/21 (23.8%)	7/21 (33.3%)	18/21 (85.7%)
PCR−	0/21 (0%)	1/21 (4.8%)	2/21 (9.5%)	3/21 (14.3%)
Total	6/21 (28.6%)	6/21 (28.6%)	9/21 (42.9%)	21/21 (100%)
IgG for ORF2/2				
PCR+	1/19 (5.3%)	4/19 (21.1%)	11/19 (9.5%)	16/19 (57.9%)
PCR−	0/19 (0%)	0/19 (0%)	3/19 (4.8%)	3/19 (15.8%)
Total	1/19 (5.3%)	4/19 (21.1%)	14/19 (14.3%)	19/19 (100%)
IgG for ORF2/3				
PCR+	5/21 (23.8%)	2/21 (9.5%)	11/21 (52.4%)	18/21 (85.7%)
PCR−	0/21 (0%)	0/21 (0%)	3/21 (14.3%)	3/21 (14.3%)
Total	5/21 (23.8%)	2/21 (9.5%)	14/21 (66.7%)	21/21 (100%)
IgG for ORF1/1				
PCR+	1/18 (5.6%)	0/18 (0%)	14/18 (77.8%)	15/18 (83.3%)
PCR−	0/18 (0%)	0/18 (0%)	3/18 (16.7%)	3/18 (16.7%)
Total	1/18 (5.6%)	0/18 (0%)	5/18 (27.8%)	18/18 (100%)
IgG for ORF1/2[b]				
PCR+	0/20 (0%)	2/20 (10%)	15/20 (75%)	17/20 (85%)
PCR−	0/20 (0%)	0/20 (0%)	3/20 (15%)	3/20 (15%)
Total	0/20 (0%)	2/20 (10%)	18/20 (90%)	20/20 (100%)
IgG status for any antigen[c]	8/21 (38%)	8/21 (38%)	5/21 (24%)	21/21 (100%)

[a] Any ORF1 construct.
[b] ORF1/2ΔArg construct was used, which has the N-terminal 62 aa arginine-rich region deleted.
[c] Classified as positive if showed positive response to any of the antigens, classified as negative if showed no response to any of the antigens.

were not tested). Subject #29 showed a borderline reaction with ORF1-C, and was seronegative for all the other constructs.

Follow-up studies

Follow-up samples from four additional subjects were analyzed for TTV DNA and for TTV antibodies (Table 3). Two subjects, #2 and #80, negative for both TTV-DNA and TTV genotype-6 DNA, showed positive or borderline ORF1-IgG results, respectively. Subject #2 was also IgG positive for the ORF1/1 and ORF1/2 encoded proteins. Of note, subject #2 had been TTV-DNA positive by UTR-PCR in an earlier sample from 1994 (Kakkola et al., 2002). Two subjects had in serum genotype-6

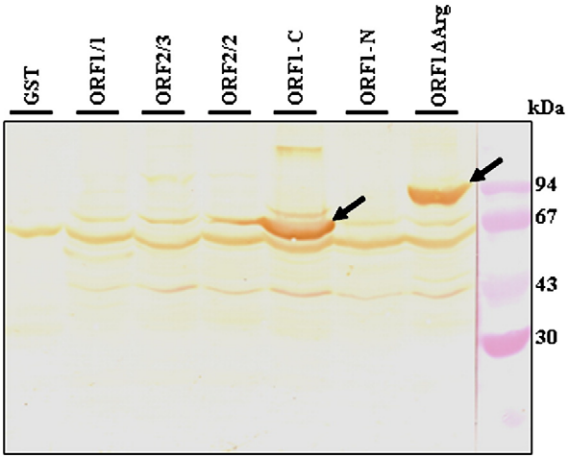

Fig. 4. Example of immunoblotting with one human serum (subject #32 of the follow-up group) showing strong IgG reactivity (arrows) against ORF1ΔArg and ORF1-C proteins.

Table 3
Follow-up of four subjects for IgG antibodies and for TTV DNA

Year	IgG for ORF1	IgG for ORF2	IgG for ORF2/2	IgG for ORF2/3	IgG for ORF1/1	IgG for ORF1/2	TTV DNA	Genotype-6 DNA
#2								
2005	+	−	−	−	+	+	−	−
2006	+	−	−	−	+	+	−	−
#32								
2000	+	−	−	−	+	−	+	+
2005	+	−	−	−	+	−	+	+
2006	+	−	−	−	+	+/−	+	+
#80								
2000	+/−	−	−	−	−	−	−	−
2005	+/−	−	−	−	−	−	−	−
2006	+/−	−	−	−	−	−	−	−
#86								
1993	−	−	−	−	−	−	+	−
1996	−	−	−	−	−	−	+	+
1998	−	+	−	−	−	−	+	+
2001	−	+	−	−	−	−	+	−
2005	−	+	−	−	−	−	+	−
2006	−	+	−	−	−	−	+	−

DNA either persistently (#32) or transiently (#86). Subject #32 was IgG positive for ORF1 and ORF1/1 throughout the follow-up. Subject #86 experienced a seroconversion for ORF2-IgG in 1998, and remained IgG positive thereafter, but was IgG negative for all the other proteins. At the time of the seroconversion, the genotype-6 DNA disappeared from circulation (Table 3).

Genotype-6 amplicons obtained and sequenced from these two subjects, showed no major changes during follow-up. Of the seven sequences obtained from the 1996 serum of subject #86, six showed the same 8/223 nucleotide differences (leading to amino acid change in three positions) and the seventh showed one additional difference (also at amino acid level) compared to our genotype-6 clone. The same 8 nucleotide differences (and one additional, but not leading to amino acid change) were present in 1998 (Kakkola et al., 2002). The sequences of subject #32 (from whom the genotype-6 clone was initially isolated in 1998) showed in 2000–2006 no nucleotide differences compared to the genotype-6 clone, or only isolated single-nucleotide changes (leading to amino acid changes only twice). This person had in 1998 at least two additional TTV isolates in circulation (Kakkola et al., 2002). The titers of genotype-6 DNA (determined by end-point dilution of PCR followed by amplicon hybridization) were stable over time: #32 and #86 had DNA titers of 10^{-1} and 10^{0}, respectively. Taken together, in subject #32 the nucleotide sequence remained stable for 8 years (from 1998 to 2006) at the same titer. Throughout this time she also had antibodies for ORF1. In subject #86 the nucleotide sequence remained stable for the two years that the genotype-6 DNA was detectable in her serum. Of note, upon disappearance from circulation of the genotype-6 DNA, the ORF2-IgG antibodies appeared.

Discussion

In order to elucidate the immunology of TTV infections, we expressed all six proteins of genotype 6, and used them as antigens in immunoblots to detect the protein-specific IgG responses in human sera.

Expression of TTV proteins

As in our previous study (Kakkola et al., 2002), the expression of the putative capsid protein, ORF1, was not successful as an entire full-length protein. Although in transfection studies the mRNA encoding this protein has constituted as much as 60% of the total RNA, it has been suggested that the amount of protein translated is low or that the

protein is susceptible to degradation (Qiu et al., 2005). However, we were able to express this protein in two slightly overlapping parts and also as an arginine-depleted construct.

In our previous study we expressed two proteins of the ORF2 region: the ORF2a and ORF2b-encoded proteins, the genes of which are separated by a stop codon and a frame shift (Kakkola et al., 2002). This stop codon interrupting ORF2 has been identified also in other, though not all, TTV isolates (Kakkola et al., 2002; Tanaka et al., 2000). Based on the transcription map (Qiu et al., 2005), it is likely that due to mRNA splicing, the ORF2a region is removed and the protein is not produced. Nevertheless, it is possible that in cells of other types or under some particular conditions this protein is expressed. The ORF2 expressed in this study is included in ORF2b, described earlier (Kakkola et al., 2002), but is 39 amino acids shorter at the N-terminus.

The ORF1/2 and ORF2/2 proteins are highly enriched in C-terminal serine residues. Asabe et al. (2001) showed that the ORF3 protein (equaling to our ORF2/2) of genotype 1a occurs in immunoblots in two forms that differ in phosphorylation state (Asabe et al., 2001). In our immunoblots from insect cells the ORF2/2-encoded protein occurred in multiple molecular sizes, possibly representing the differentially phosphorylated forms. Whether similar phosphorylation occurs on the ORF1/2-encoded protein is not known. In database searches TTV proteins have shown resemblance to other proteins: Takahashi et al. (2000) noted in serine-rich proteins of TTV and TTMV similarities with topoisomerase (Takahashi et al., 2000), and Kamahora et al. (2000) found in the ORF2-4 protein of genotype 1 (equaling to our ORF2/2) similarities with transcription factors and RNA-binding motifs (Kamahora et al., 2000). We were able to express the ORF2/3-encoded protein in high amounts both in bacteria and in insect cells. This protein equals to the ORF2–5 protein reported by Kamahora et al. (2000), which also has been predicted to share properties with transcription factors (Kamahora et al., 2000). However, no definitive function has yet been assigned to any of these proteins.

In expression of the ORF1 and ORF1/2-encoded proteins, we noted that the presence of the arginine-rich N-terminus (aa 1–62) reduces or abolishes protein expression. The Rosetta strain of *E. coli* which co-expresses tRNA for arginine codons, did not increase the yield. The expression levels were however dramatically increased upon deletion of this arginine-rich part. The expression of ORF1/1 was less successful, for an unknown reason.

Among TTVs, additional splicing events and intragenomic rearrangements have been suggested to form yet additional ORFs (Leppik et al., 2007). It is thus possible that different genotypes/genogroups of TTV show differences in mRNA splicing and/or protein expression. In addition, the transcription profile could vary between cell types; for example the activity of the promoter region seems to differ in various cell types (Kamada et al., 2004; Suzuki et al., 2004). However, the occurrence of additional mRNAs and ORFs, potentially leading to new proteins, remains to be shown.

The prevalence of IgG

Based on previous reports, it is evident that some degree and form of TTV immune activity occur in humans. IgG antibodies have been shown by immunoprecipitation in some subjects after viremia (Tsuda et al., 1999). By immunocapture combined with PCR, the kinetics of IgM and IgG antibodies have been shown to follow the classical course, i.e. short-lived IgM followed by long-lasting IgG (Tsuda et al., 2001). TTV in serum has been found as immunocomplexed with IgG (Itoh et al., 2000), especially during persistent infections (Nishizawa et al., 1999). Interestingly, superinfection with a new TTV strain decreased the immunocomplexes, and was followed by clearance of the superinfecting strain. It was discussed that superinfections, as opposed to primary/persistent infections, might be more effectively cleared by preformed antibodies (Maggi et al., 2006). It has also been estimated that over 90% of the TT viruses in circulation are cleared and replenished daily by progeny viruses, thus indicating chronic active infection (Maggi et al., 2001). It is evident that humans acquire TTV very early in life; even transplacental transmission has been suggested (Gerner et al., 2000; Matsubara et al., 2001; Morrica et al., 2000; Saback et al., 1999). Infection during immune development could also help the virus to establish persistence. Nevertheless, experimental data on immune responses towards TTV is very scanty.

In this study we have detected with immunoblots in human sera IgG antibodies against all the TTV proteins, suggesting that they all are produced *in vivo*. The antibody pattern was different in each individual, for an unknown reason. Ott et al. (2000) found a nearly 100% prevalence of antibodies towards the C-terminus of ORF1 (Ott et al., 2000); in comparison, our ORF1 IgG prevalence of 48% (including 33% borderlines) was much lower. This could either reflect an immunological difference between the TTV genotypes (genotype 1 vs. genotype 6) or be due to the methods used. The IgG prevalence of 38% detected by Handa et al. (2000) using as antigen the N-terminus of genotype-1 ORF1 (Handa et al., 2000), is in the same order of magnitude as our overall ORF1-IgG prevalence. Contradictory to that, we did not detect antibody reactivity against the N-terminus; instead, all our responses against the ORF1 encoded protein targeted the C-terminus. It remains to be seen whether the immunological differences are genotype-specific. The IgG results obtained with the ORF2 antigen in this study correlated with the antibody responses detected in our previous study (Kakkola et al., 2002), thereby further confirming the specificity of the IgG reactivities against the ORF2 protein.

In our previous studies we have shown that the genoprevalence of genotype 6 in Finland is 4–8.6% (Kakkola et al., 2002; Kakkola et al., 2004). In this study 44% of our subjects had IgG antibodies towards one or more of the TTV genotype-6 proteins. Were TTV antibodies genotype-specific, this seroprevalence could indeed reflect the cumulative lifetime infection incidence of this particular virus type in the Finnish population.

Follow-up study

We have previously (Kakkola et al., 2002) identified three genotype-6 positive subjects, two of whom were here characterized further. Subject #32 had been genotype-6 DNA positive for at least 8 years (1998–2006). This subject was strongly IgG positive for the ORF1 and ORF1/1 proteins for at least six years. Of note, the full-length clone from which the proteins were expressed in this study had been isolated from her serum in 1998. We wished to see whether the genotype-6 DNA sequence would change during these years. We found no major nucleotide changes, suggesting that the TTV sequence of the amplified region remains unaltered regardless of functional B-cell immunity. Interestingly, also the relative level of genotype-6 DNA in her sera remained constant. Subject #86 was followed for 13 years. Through these years she was positive for TTV DNA in general, and experienced a temporary genotype-6 viremia, after which she seroconverted to ORF2 IgG.

The ORF1 protein has been shown to contain within the amino acids 275–402 hypervariable regions (HVRs) which have been suggested to aid the virus in immune evasion and in establishment of persistent infections (Jelcic et al., 2004; Luo et al., 2002; Nishizawa et al., 1999; Umemura et al., 2002). Our amplicon sequence of nucleotides 1918–2143 (amino acids 447–522) was outside of these HVRs. Thus within the HVRs more mutations could possibly have occurred than within the region observed here. Published data on alterations in TTV sequences among persistently infected subjects are controversial. It has been proposed that, during TTV persistence the sequences change, isolates are cleared from circulation and replaced by reinfection, or sequences remain the same, thereby indicating either true persistence, and/or superinfections, as well as evolution of quasispecies (Ball et al., 1999; Biagini et al., 1999; Gallian et al., 1999;

Irving et al., 1999; Wilson et al., 2001). Interestingly, Nishizawa et al. (1999) suggested that in acute infection quasispecies do not exist, while in chronic infection TTV sequences continuously evolve (Nishizawa et al., 1999). Our follow-up data indicate that genotype-6 viremias can be either persistent or transient, with no nucleotide changes within the amplified region of ORF1.

Conclusions

TTV is a minute virus, obviously lacking the genetic capacity of the larger viruses for immune evasion. Nevertheless, TTV seems to be adapted to its host very well, whereby the long-term virus-host co-evolution could explain the existence of the numerous TTV genotypes and the possible lack of strong immune responses. In order for us to achieve a full picture of TTV and its interplay with our immunity, also proteins of other genotypes need to be expressed and analyzed for antibody isotypes and cross-reactivity, for conformational and linear epitopes, and for affinity/avidity. In addition, the role of T-cell function in TTV infections needs to be elucidated. To begin with, we have expressed all six proteins of TTV genotype 6, and shown them to be immunoreactive with human antibodies.

Materials and methods

The transcription map of the full-length genotype-6 plasmid clone has been determined in transfected 293 cells (Qiu et al., 2005) and is shown in Fig. 1. The primers for (RT-)PCRs to obtain the cDNA constructs for the expression of the proteins were designed based on the transcription map. Nucleotide numbering is according to the TTV HEL32 sequence, GenBank # AY666122.

Construction of expression plasmids

The cDNA constructs for protein expression in bacteria and in insect cells were obtained with PCR and/or RT-PCR: ORF1 (nt 581–2791), ORF1ΔArg (nt 767–2791, aa 63–737), ORF1-N (nt 581–1787, aa 1–402), ORF1-NΔArg (nt 767–1787, aa 63–402), ORF1-C (nt 1613–2791, aa 344–737), ORF2 (nt 354–707), ORF1/1ΔArg (nt 689–703 and 2315–2791, aa 37–199) and ORF1/2ΔArg (nt 689–703 and 2505–2810, aa 37–142), ORF2/3 (nt 354–703 and 2505–2982), ORF1/2 (nt 581–703 and 2505–2810), ORF2/2 (nt 354–703 and 2315–2810), and ORF1/1 (nt 581–703 and 2315–2791). For termination of translation, the stop codon of the viral gene was utilized (i.e. the primer was designed down stream of the stop codon or the primer overlapped with the viral stop codon). Table 4 lists the primers used for cloning of the cDNAs into expression plasmids.

PCRs to produce and amplify the cDNAs were done either with Ampli Taq Gold (Applied Biosystems/Roche) or with the Expand High Fidelity PCR system (Boehringer Mannheim/Roche) as recommended by the manufacturer, and the templates were either the TTV genotype-6 plasmid clone (pTTV) (Kakkola et al., 2007) or the cloned ORFs.

RT-PCRs to amplify the cDNAs were done either with OneTube Titan RT-PCR System (Roche) or by RobusT II RT-PCR Kit (Finnzymes) as recommended by the manufacturer, and the template was total RNA isolated on day 3 from pTTV-transfected 293 or 293T cells (Kakkola et al., 2007; Qiu et al., 2005).

The cloning of cDNAs into expression plasmids was done in *E. coli* strain DH5α with standard restriction enzyme and ligation protocols. For expression in bacteria, the cDNAs were cloned into pGEX-4T-1 (Amersham Pharmacia), and for expression in insect cells, the cDNAs were cloned into pAcGHLT-A Baculovirus Transfer Vector (BD Biosciences). The plasmid clones were isolated from the bacteria with QIAprep Spin Miniprep Kit (Qiagen) and with GenElute Endotoxin-free Maxiprep Plasmid Purification Kit (Sigma), respectively.

All the plasmid clones were sequenced and confirmed to be in frame with fusion proteins and to contain exactly the same nucleotide sequence as in the TTV genotype-6 isolate HEL32 (GenBank accession number AY666122). The sequencing reactions were done using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems) at the sequencing core facility of the Haartman Institute, University of Helsinki, Finland.

Table 4
Primers used for cloning the open reading frames into expression plasmids

Primer name (and orientation)	5′–3′ primer sequence (nt numbering according to AY666122)	Used for amplification of
noARG (forward)	ttt**gaattc**ATGcgcagacacagaaaaaaac (nt 767–785)	ORF1ΔArg, ORF1-NΔArg
RepCF (forward)	ttt**gaattc**aacacatggtacagaggcaatg (nt 1613–1634)	ORF1-C (bacteria)
RepCR (reverse)	ttt**cccggg***tta*tgcatgggaagatagt (nt 2791–2773)	ORF1, ORF1ΔArg, ORF1-C (insect cells)
3F (forward)	tcg**gaattc**ATGgcctggtactggt (nt 581–596)	ORF1, ORF1-N, ORF5 (outer PCR), ORF6
O1aSTOP (reverse)	ttt**cccggg***tta*tcctggggacaggaatatg (nt 1786–1768)	ORF1-N, ORF1-NΔArg
O1endATG (forward)	ttt**gaattc**ATGaacacatggtacagaggcaatg (nt 1613–1634)	ORF1-C (insect cells)
Nsf (forward)	ttttt**gaattc**ATGtggcagccacctacccag (nt 354–374)	ORF2, ORF3 (outer PCR), ORF4
2R (reverse)	tctaataaag**gcggccgc**ccactg (nt 802–797)	ORF2
Ns1rkor (reverse)	ttttt**cccggg***tta*aacataaagaccttgtt (nt 2810–2791)	ORF3 (outer PCR), ORF6, ORF6ΔArg
ORF3srkorj (reverse)	ctggagaaagtgtaagagcatctg (nt 2504–2481)	ORF3 (inner PCR), ORF5 (inner PCR)
ORF3sf (forward)	gccgcagaatcgtccgac (nt 696–703 →2314–2324)	ORF3 (inner PCR), ORF5 (inner PCR)
Ns2r (reverse)	ttttt**cccggg***tta*atgaaagccaagtttg (nt 2979–2964)	ORF4
3R (reverse)	gctttgggca**gcggccgc**tatgtgg (nt 2921–2915)	ORF5 (outer PCR), ORF5ΔArg
noARG2 (forward)	ttt**gaattc**ATGcgcgctcgccgcaga (nt 689–703)	ORF5ΔArg, ORF6ΔArg

The restriction sites are shown with bolded letters, the translation initiation codons with capital letters and the translation stop codons with italics. Nucleotide numbers correspond to the sequence complementary to AY666122, thus excluding restriction enzyme sites and 5′ upstream sequences.

Expression of proteins in bacteria

For prokaryotic expression, TTV genotype-6 cDNAs were cloned in fusion with a glutathione S-transferase protein (GST-protein). For protein expression, the plasmid clones were transformed into *E. coli* strain BL21, and for the expression of arginine-rich proteins, into *E. coli* strain Rosetta (Novagen). The bacteria were grown over night in L-Broth medium at +37 °C, followed by 2 h at +37 °C as diluted 1:100. Protein expression was then induced with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4–6 h. Optimization of the expression conditions was done with the constructs that showed low level expression with our standard expression conditions; when necessary, growth time after dilution (2–6 h) and induction times (1–6 h) (marked as e.g. 5/2 in Table 1), induction temperatures (room temperature or +37 °C), and growth media (L-Broth, YT or Terrific Broth) were optimized. The expressed proteins were analyzed with SDS-PAGE and immunoblotting.

Expression of proteins in insect cells

For eukaryotic expression, TTV genotype-6 cDNAs were cloned in fusion with GST and with hexahistidine (GST-6xHis-tag). *Spodoptera frugiperda* (Sf9) insect cells were transfected by Fugene-6 Transfection Reagent (Roche) with Linearized Baculovirus DNA (BD Biosciences) and with the vector containing the TTV-cDNA insert. The transfected Sf9 cells produced recombinant baculoviruses encoding the GST-6xHis-fusion proteins. For large scale protein expression, Sf9 and/or *Tricoplusia ni* (HighFive) insect cells were infected with recombinant

baculoviruses. The expressed proteins were analyzed with SDS-PAGE and immunoblotting.

Immunoblotting

The proteins of the crude cell lysates were separated on 10% SDS-PAGE, and transferred to Protran nitrocellulose membranes (Schleicher & Schüell) at 15 V for 30 min with Trans-Blot SD Electrophoretic Transfer Cell (Bio-Rad). The membranes were blocked with 5% fat-free milk powder (Valio) and 0.2% Triton X-100 in PBS. For immunodetection of the GST fusion proteins, the primary antibody was mouse α-GST Ab-1 (LabVision) in 1:445 dilution, and the secondary antibody was peroxidase-conjugated rabbit α-mouse IgG (DAKO) at dilution 1:500.

For analysis of TTV-specific IgG antibodies in human sera, the samples were diluted 1:50 in PBS containing 5% milk powder and 0.2% Triton X-100 and were used as primary antibody. The secondary antibody was peroxidase-conjugated rabbit α-human IgG (DAKO) diluted 1:500.

Bound antibodies were detected with hydrogen peroxide and diaminobenzidine (DAB). Bacteria expressing the sole GST, or insect cells infected with the GST-6xHis-recombinant baculovirus were used as positive controls for expression, and as negative controls in immunoblotting in addition to the uninduced or non-infected cells.

Serum samples

Sera were obtained, with informed consent, from 25 non-symptomatic Finnish adults (Kakkola et al., 2002). In addition, sequential serum samples were obtained from four individuals. This study was approved by the Ethical Committee of the Helsinki University Central Hospital.

Universal PCR and genotype-6 PCR

Isolation of DNA from sera, universal PCR (UTR-PCR) and genotype-6 PCR were done as described previously (Kakkola et al., 2002). The amplicons obtained from the genotype-6 positive sera were purified with High Pure PCR Product Purification Kit (Roche), and cloned into pSTBlue-1 AccepTor Vector (Novagen) in *E. coli* DH5α. Of each amplicon, 4–7 bacterial clones containing a genotype-6 sequence were isolated and sequenced. One amplicon was also sequenced directly from the genotype-6 PCR. The amount of genotype-6 DNA in sera was semi-quantified with end point dilution of the PCR template. For sensitivity and specificity, the PCR amplicons were additionally analyzed by Southern hybridization with a digoxigenin (DIG) -labelled PCR-generated probe (Kakkola et al., 2002).

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